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BRIEF COMMUNICATION

Genetic variation within and among small isolated populations of *Santalum album*

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Abstract

A combination of directed amplification of minisatellite DNA (DAMD) and random amplification of polymorphic DNA (RAPD) primes were used to assess the genetic variation within and between three isolated populations of Indian sandalwood (*Santalum album*). Eleven primers used in this study amplified 65.99 % polymorphic bands. Analysis of molecular variance revealed a high genetic variation among these populations ($\phi_{ST} = 0.549$). There are indications of clonality within the existing Indian sandalwood populations which can be attributed to habitat fragmentation, isolation and vegetative reproduction.

Additional key words: DAMD, genetic diversity, population fragmentation, RAPD, sandalwood.

The sandalwood populations in India have been subjected to indiscriminate felling over several decades. Conservation of genetic resources requires a thorough evaluation of existing diversity in germplasm. A large number of methodologies exist for the assessment of genetic diversity in plant species. Previous studies have suggested that *Santalum album* in India has several genetic hotspots and a wide genetic base (Shashidhara *et al.* 2003, Rao *et al.* 2007). We selected three representative fragmented populations from the southern interior region of India for this study.

Despite their tendency to underestimate intra-population diversity because of dominance and biallelism, RAPDs are tailor made to detect variations in the genome due to sequence duplications and accumulated errors of replication (Aagaard *et al.* 1998). The RAPDs have proved to be important trait linked markers in horticulture crops like guava and sugar beet (Amiri *et al.* 2009, Fereira-Romero *et al.* 2009). DAMD markers are evenly distributed in the plant genome and exhibit relatively higher mutation rate (10^{-3}) (Tourmente *et al.* 1998). A relatively higher stringency is the major advantage of minisatellite markers. Hence, pooling of RAPD and DAMD data can be very effective in studying intra specific variation at the DNA level.

Sandalwood (*Santalum album* L.) trees were sampled from three different locations of Southern India *viz.,* Kunigal (KN, 11° 27′ N, 77° 26′ E), Kengeri (BU, 13° 01′ N, 77° 01′ E) in Karnataka and Kallipatti (KP, 12° 55′ N, 77° 29′ E) in Tamil Nadu. They were selected based on the criteria of age, distance between successive samples and phenotypic differences. Leaves (2 - 3 g) were collected from each tree, frozen in liquid nitrogen and stored at -80 °C. DNA was extracted using CTAB extraction protocol (Doyle and Doyle 1990). Quantified and diluted DNA was used for RAPD-PCR (94 °C for 4 min and 45 cycles of 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min followed by 10 min final extension at 72 °C) with 7 independent random decamer primers. Amplification was performed in duplicates in a 0.025 cm^3 reaction volume containing 35 ng of DNA template; 0.0025 cm^3 10× buffer (3-trishydroxymethyl-methyl-amine-propane-sulphonic acid), pH 8.8, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01 % gelatin, 2.5 mM each of dATP, dCTP, dTTP, dGTP, 0.02 mM of primer, 0.3 U *Taq* polymerase. The PCR products were separated by 1.5 % agarose gel electrophoresis (60 V) with 1× TAE buffer (100 mM Tris-HCl, pH 8.3, 83 mM acetic acid, 1 mM EDTA) followed by ethidium bromide staining

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Abbreviations: AMOVA - analysis of molecular variance; DAMD - directed amplification of minisatellite DNA; RAPD - random amplification of polymorphic DNA.

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and UV irradiation. The size of the amplified products was compared to pBluescript *Hin*f I and lambda DNA *Hin*d III digested markers. DAMD primers (RMS1 - RMS 4) were used for stringent amplification (50 °C) of minisatellite core sequences (Zhou *et al.* 1997, Bhattacharya and Ranade 2001). Consistent and unambiguous amplicons were scored as present (1) or absent (0) for each sample. Data from all RAPD and DAMD amplifications was combined. Homology and distinctness of the bands was ascertained by the co migration of the bands on the gel.

The Dice pair wise distances were calculated using *RAPDALG* program from *RAPDistance* software (Dice 1945, Nei and Li 1979). A neighbour-joining (NJ) tree with distance values was generated using *NJTREE.EXE* and *TDRAW.EXE*. The *SEQBOOT* program of *PHYLIP* was run (1000 replications) to assess the strength of the test statistics. The phylogeny estimate was performed by *PARS*, which gives a list of equally parsimonious trees with different branch lengths (Hochbaum and Pathria 1997). The consensus tree obtained by the *CONSENSE* program of *PHYLIP* was displayed with *TREEVIEW* software. The extent of population subdivision was examined using analysis of molecular variance. *WINAMOVA 1.04* was used to calculate Φ-statistics and null distribution of pair-wise Φ_{ST} values was obtained by 1000 permutations of the original data set.

Table 1. Primer sequences, the total number of bands, number and percentage of polymorphic bands (PB) amplified using RAPD and DAMD primers.

Primer	Sequence	Total	PB	[%]
OPA01	CAGGCCCTTC	24	17	70.84
OPB08	GTCCACACGG	16	11	68.75
OPD12	CACCGTATCC	19	14	73.68
OPE02	GGTGCGGGAA	15	11	73.34
OPF05	CCGAATTCCC	24	14	58.34
OPG01	CTACGGAGGA	24	16	66.67
OPH05	AGTCGTCCCC	17	13	76.47
RMS1	GGCGGAGGTGGCCC	17	8	47.05
RMS2	GGAGGTTTTCA	23	14	60.86
RMS3	GGTGTAGAGAGAGGGGT	21	15	71.42
RMS4	CCTCCTCCCTCCT	20	12	60.00

RAPD and DAMD primers together consistently amplified 220 scorable bands ranging from 200 to 2000 base pairs (Table 1). Our results show that *S. album* has a high number of polymorphic loci (65.9 %) as observed in many woody tree species with similar life cycles (Lacerda *et al.* 2001, Shrestha *et al.* 2002). *AMOVA* revealed a high ϕ_{ST} value of 0.549 for variance among populations (Table 2), which is typical of insular systems with physical barriers to contain the gene flow. Microsatellite marker studies on some endemic species of *Santalum* from the pacific islands show similar ϕ_{ST} values (Bottin *et al.* 2005, Lhuillier *et al.* 2006). Although, the molecular markers used in those studies are different from that of ours, there are several points that make this comparison reasonable. It is supposed that sandalwood in India has its origin in Indonesian island of Timor (Shetty 1977). There is molecular evidence to show that generally island populations exhibit lower genetic variations than their counterparts on the mainland (Frankham 1997). Previously reported allozyme marker based heterozygosity estimates of the Timor [H_0 = 14 %] and Indian [H_0 = 31 %] sandalwood populations may apparently be different (Brand *et al.* 1994, Rao *et al.* 2007). However, it is also true that the higher genetic diversity of the species in India is largely due to a bigger population size and distribution range.

The Dice pairwise distances used to develop a NJ tree dendrogram (Fig. 1) clearly depicted genetically diverse tree populations with clonality within the populations. The *BOOTSTRAP* values were less than 50 % within all the populations and 100 % for the populations from Tamil Nadu and Karnataka, which is in consonance with the geographical topology. These high *BOOTSTRAP* values further endorse the sampling strategy wherein all the three populations exhibit affiliations to their closest geographical allies. There are several indicators that show the tendency of Indian sandalwood populations to possess lesser genetic variation than an average indigenous mainland plant species. The populations used in the present investigation are partly mimicking the insular systems because they are subjected to isolation by natural and human interference. A high variance among populations (54.9 %) suggests a restricted gene flow.

Loss of diversity has taken center stage in population ecological research during last couple of decades (Stockwell 2003). Habitat fragmentation is a major factor that reduces population fitness. It creates genetic bottlenecks as the remaining trees contain only a small sample of the original gene pool. Many studies have shown that fragmented and isolated populations of out-breeding species tend towards selfing and conservation efforts have to specifically target such species (Aguilar

Table 2. Analysis of molecular variance (*AMOVA*) for 65 individuals in three populations of *Santalum album* (ϕ_{ST} = 0.549).

	Sum of squares	Degrees of freedom	Mean squares	Variance components	Total [%]
Among populations	1176.66	2	588.33	27.86	54.9
Within populations	1419.16	62	22.89	22.88	45.1

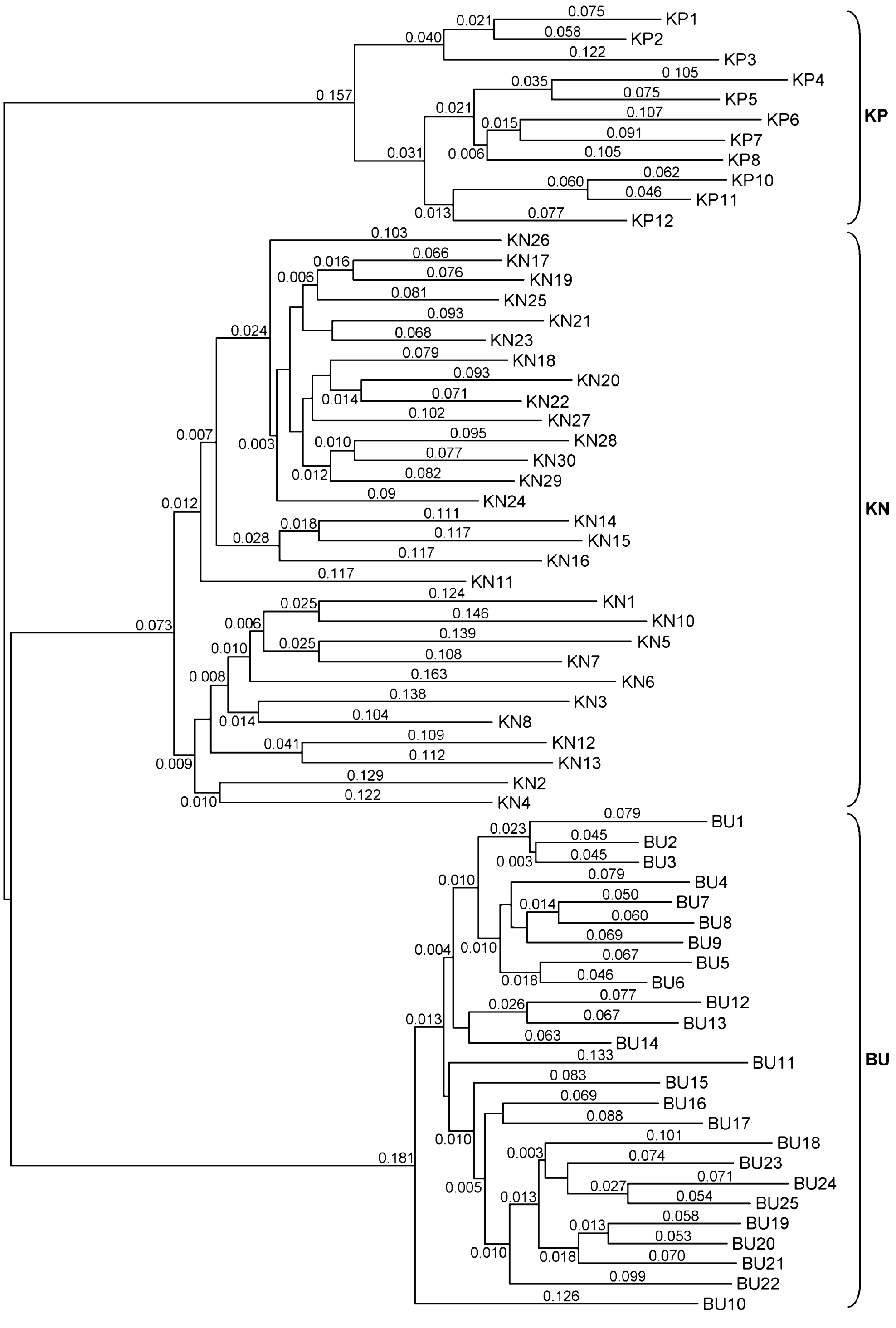

Fig. 1. Dendrogram generated for the 65 individuals in three populations of sandalwood by means of the NJ method based on Dice distance using *RAPDistance* program.

et al. 2008). *Santalum* is a cross pollinated species and shows a broad area of distribution. Sandalwood population dynamics needs a closer examination because its numbers are decreasing alarmingly along with reduction in habitat. Our study clearly shows that small isolated pockets of sandalwood plantations in India are unique and tending towards clonality (*BOOTSTRAP* re-sampling analysis). These initial leads can be utilized further to develop new conservation strategies for sandalwood management.

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